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13. ABSTRACT *(Maximum 200 Words)*

This concept award aimed to identify endogenous BRCA1 phosphorylation sites using a new technology based on mass spectrometry as a prelude for the analysis of regulation of BRCA1 by phosphorylation. We made great effort to isolate sufficient amount of the BRCA1 protein from cycling HeLa cells so that we can analyze phosphorylation of the protein and identify the exact phosphorylation sites by mass spectrometry. BRCA1 is so scarce that we can only purify ~ 200 ng of the protein from ~ 50 L HeLa cells. Despite of the power of mass spectrometry, this amount of protein (~ 1 pmol) only allowed us to identify a single phosphorylation site by mass spectrometry each time. We managed to identify one in vivo phosphorylation site as S1189. S1189 does not confer to phosphorylation consensus of any known BRCA1 kinases, suggesting that BRCA1 is subject to regulation by a novel BRCA1 kinase. Mutagenesis of this site and complementation of the HCC1937 cell lines are underway to assess the functional significance of this phosphorylation site.

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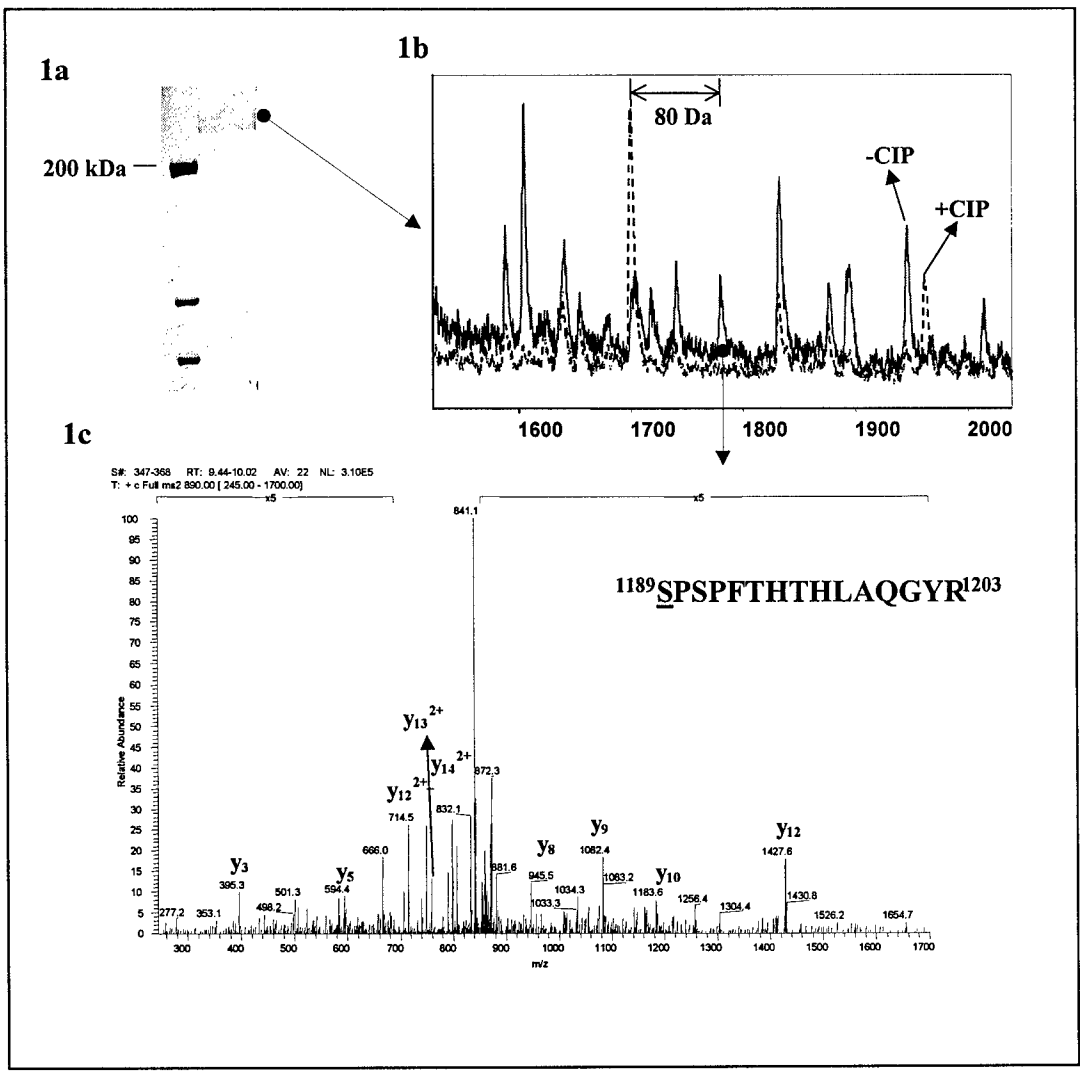
Introduction

Understanding the role of phosphorylation of BRCA1 will be the next logical step for a complete understanding of BRCA1 functions. The identification of in vivo phosphorylation sites is therefore the prerequisite for this effort. It has been technically changeling with traditional techniques to identify in vivo phosphorylation sites for the BRCA1 protein, as it is not an abundant protein. However, recent advance in mass spectrometry has made this task within reach. I proposed to identify in-vivo phosphorylation sites of the endogenous BRCA1 protein without exogenous DNA damage from asynchronized cells and study their functional significance by site-directed mutagenesis and complementation of the breast cancer cell line HCC1937, in which the BRCA1 protein is truncated and its expression is much lower than normal cells. Identification of in vivo phosphorylation sites and investigation of functional significance of these phosphorylation sites will open new directions for unraveling the tumor suppressor function of BRCA1, and ultimately mechanisms by which BRCA1 suppresses tumor formation in breast tissues.

Body

We carried out experiments as proposed in the grant. Since this is a short one year grant, aiming at acquiring preliminary data and insight that may point to a new direction for breast cancer research, much of our work is at the exploration of the feasibility of using a new technique that is based on mass spectrometry to identify in vivo phosphorylation sites of BRCA1. Since BRCA1 is not abundant, it has been proven very difficult to identify phosphorylation sites by conventional 2D peptide mapping with P^{32} labeling.

Key Research Accomplishments



1. Identification of phosphorylation sites with mass spectrometry

The identification of in vivo phosphorylation sites using mass spectrometry is a multiple step process (8). The phosphoprotein is separated by SDS-PAGE, and then in-gel digested with trypsin. The resulting peptides are recovered by extraction with organic solvent and subject to MS analysis. Phosphopeptides are identified with a combination of phosphatase treatment followed by mass spectrometric measurement, in which a loss of 80 Da in mass is observed. The phosphopeptides are then sequenced by collision induced dissociation (CID) in a liquid capillary chromatography (LC)/MS /MS instrument to pinpoint the amino acid residue that is phosphorylated. For example, in our preliminary analysis of phosphorylation sites of BRCA1, BRCA1 was immunoprecipitated from HeLa nuclear extract (Fig.1a). The band was then processed and a matrix assisted laser desorption time of flight mass spectrum was taken (Fig.1b). From the phosphatase treated and untreated samples, a phosphopeptide was identified. This phosphopeptide was then sequenced by CID in a capillary HPLC/MS/MS system (Fig 1c). Analysis of the CID spectrum thus pinpoints the phosphorylation site as S1189.

To circumvent the problem of purifying endogenous BRCA1 protein, we also investigated over-expression of BRCA1 in 293T cells. We could purify more BRCA1 proteins this way, the phosphorylation stoichiometry is lower, probably because of the endogenous kinases cannot keep up with the over-expressed proteins, therefore over-expression may not be a viable alternative.

Reportable Outcomes

We have identified one in vivo phosphorylation site S1189 of BRCA1. Since this site does not confer to consensus phosphorylation site of known BRCA1 kinases, we suspect that a novel kinase regulates BRCA1 by phosphorylation.

The reviewer was puzzled when reviewing our original report submitted in April that S1189 was reported as an in vivo phosphorylation site of BRCA1 on the paper that we published in Science (1999, 286: 1162-66). S1189 was identified in an experiment in which BRCA1 was purified from transient co-transfection of BRCA1 and ATM cDNA in 293T cells followed by γ irradiation. This was a recombinant BRCA1, not the endogenous BRCA1. This is different from the experiment that is discussed in the current final report. We purified endogenous BRCA1 from cycling HeLa cells that was not irradiated.

Technically, it is much more challenging to purify endogenous proteins than recombinant proteins. This is partly the reason that we could not identify more in vivo phosphorylation sites of BRCA1 as we have hoped. The technical difficulty lies in the extremely low abundance of the BRCA1 protein. The reason that we identified S1189 again from the endogenous BRCA1 protein is probably pure technical. Some peptides are better ionized and detected in mass spectrometry than others. The mechanism is not very clear, but it is well known in the mass spectrometry field. The peptide spanning a. a. 1189 to 1203 happens to be such a peptide. Despite that we encountered difficulty and failed several times in identifying more in vivo BRCA1 phosphorylation site, mass spectrometry is still the best method that is currently available to biological researchers.

We did attempt to analyze functional significance of S1189 phosphorylation. Our proposed approach using retrovirus to complement HCC1937 cells has proven difficult. Since BRCA1 is a rather large gene ~ 5.5 kb, it proved not trivial to sub-clone it into a retrovirus vector. We managed to make the mutation and clone it in a retrovirus vector, and subsequent transduction of HCC1937 cells yielded several clones that were resistant to antibiotic selection. Unfortunately, these clones did not express the BRCA1 mutant, or expressed the protein in a very low level that could not be detected by Western blotting. We are in the process of optimizing the virus transduction protocol and generating higher tier virus for such transduction. We were successful in using this approach in the science paper. The complementation of HCC1937 with WT and mutant BRCA1 is the prerequisite for the functional analysis of S1189 phosphorylation.

Finally, a small technical point: the definition of *in vivo*. In molecular biology and cell biology, it is customary that cell culture is considered as *in vivo* and experiments carried out in a test tube as *in vitro*.

Since we purified the protein from human cell lines. It can be considered as *in vivo* sample. Laboratory animals will constitute a physiological system.

Conclusions

It is feasible to identify in vivo phosphorylation sites of BRCA1 with new technology based on mass spectrometry. However, it is not easy and requires extensive technology expertise. The preliminary data acquired will allow us to test the functional significance of the phosphorylation site. If this phosphorylation site is functional significant, it will set the stage to identify the kinase that phosphorylates this site to dissect the signal transduction pathway.

References

None

Appendices

None

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REPLY TO
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